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# Bradykinin, But Not Muscarinic, Inhibition of M-Current in Rat Sympathetic Ganglion Neurons Involves Phospholipase C- $\beta$ 4

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Rat superior cervical ganglion (SCG) neurons express low-threshold noninactivating M-type potassium channels ( $I_{K(M)}$ ), which can be inhibited by activation of  $M_1$  muscarinic receptors ( $M_1$  mAChR) and bradykinin (BK)  $B_2$  receptors. Inhibition by the  $M_1$  mAChR agonist oxotremorine methiodide (Oxo-M) is mediated, at least in part, by the pertussis toxin-insensitive G-protein  $G_{\alpha_q}$  (Caulfield et al., 1994; Haley et al., 1998a), whereas BK inhibition involves  $G_{\alpha_q}$  and/or  $G_{\alpha_{11}}$  (Jones et al., 1995).  $G_{\alpha_q}$  and  $G_{\alpha_{11}}$  can stimulate phospholipase C- $\beta$  (PLC- $\beta$ ), raising the possibility that PLC is involved in  $I_{K(M)}$  inhibition by Oxo-M and BK. RT-PCR and antibody staining confirmed the presence of PLC- $\beta$ 1, - $\beta$ 2, - $\beta$ 3, and - $\beta$ 4 in rat SCG. We have tested the role of two PLC isoforms (PLC- $\beta$ 1 and PLC- $\beta$ 4) using antisense-expression constructs. Antisense constructs, consisting of the cytomegalovirus promoter driving antisense cRNA

corresponding to the 3'-untranslated regions of PLC- $\beta$ 1 and PLC- $\beta$ 4, were injected into the nucleus of dissociated SCG neurons. Injected cells showed reduced antibody staining for the relevant PLC- $\beta$  isoform when compared to uninjected cells 48 hr later. BK inhibition of  $I_{K(M)}$  was significantly reduced 48 hr after injection of the PLC- $\beta$ 4, but not the PLC- $\beta$ 1, antisense-encoding plasmid. Neither PLC- $\beta$  antisense altered  $M_1$  mAChR inhibition by Oxo-M. These data support the conclusion of Cruzblanca et al. (1998) that BK, but not  $M_1$  mAChR, inhibition of  $I_{K(M)}$  involves PLC and extends this finding by indicating that PLC- $\beta$ 4 is involved.

**Key words:** M-current; muscarinic receptor; bradykinin; phospholipase C- $\beta$ ; antisense; superior cervical ganglion neuron

The M-type potassium current ( $I_{K(M)}$ ) is a noninactivating potassium current present in various peripheral and central neurons, including rat superior cervical ganglion (SCG) neurons (for review, see Brown, 1988). It is activated in the subthreshold range for action potentials and increases with membrane depolarization and may, therefore, be involved in controlling cell excitability, because inhibition of this current results in depolarization and increased action potential discharge.  $I_{K(M)}$  in SCG can be inhibited by stimulating various receptors including the  $M_1$  muscarinic receptor ( $M_1$  mAChR; Marrion et al., 1989; Bernheim et al., 1992; Hamilton et al., 1997) and bradykinin (BK)  $B_2$  receptor (Jones et al., 1995), both of which couple via *Bordetella pertussis* toxin (PTX)-insensitive GTP-binding proteins (G-proteins). We have previously demonstrated that the  $\alpha$  subunit of  $G_q$  mediates inhibition by  $M_1$  mAChR agonists (Haley et al., 1998a) and that  $G_{\alpha_q}$  and/or  $G_{\alpha_{11}}$  is required for inhibition by BK (Jones et al., 1995). Because both  $G_{\alpha_q}$  and  $G_{\alpha_{11}}$  are known to stimulate PLC- $\beta$  (Singer et al., 1997), we have now used antisense directed at two PLC- $\beta$  isoforms (PLC- $\beta$ 1 and PLC- $\beta$ 4) to deplete the cells of these enzymes and so determine whether either is required for inhibition of  $I_{K(M)}$  by  $M_1$  mAChR agonists or BK.

Some of this data has been previously presented in abstract form (Haley et al., 1998b)

## MATERIALS AND METHODS

**DNA plasmids.** The constructs used in this study were made by PCR cloning using standard molecular techniques (Abogadie et al., 1997). Primers that were deemed specific for each target PLC- $\beta$  isoform were used in PCR, and the products were TA-cloned into pCR3 or pCR3.1 (Invitrogen, San Diego, CA). Antisense orientation was confirmed by sequencing. The clones are as follows, in 5' to 3' orientation [nucleotide (nt); plus and minus signs indicating downstream and upstream, respectively, of the stop codon]: PLC- $\beta$ 1 antisense (clone 239-8), nt +22 to +172; PLC- $\beta$ 2 antisense (clone F128-5), nt -536 to -309; PLC- $\beta$ 3 antisense (clone E92-18), nt -93 to +67; PLC- $\beta$ 4 antisense (C58-13), nt +5 to +386.

**Cell culture.** Sympathetic neurons were isolated from SCG of 15- to 19-d-old Sprague Dawley rats and cultured using standard procedures as described previously (Delmas et al., 1998).

**Microinjection.** DNA plasmids, purified using maxiprep columns (Qia-gen, Hilden, Germany), were diluted to 400  $\mu$ g/ml in calcium- and glucose-free Krebs' solution (290 mOsm/l; pH 7.3) containing 0.5%

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FITC-dextran (70,000 MW) and pressure injected into the nucleus of SCG neurons 2 d in culture using an Eppendorf microinjector (Hamburg, Germany). Cells were maintained in culture for a further 2 d, and a survival rate of 75–85% was obtained.

**Electrophysiology.**  $I_{K(M)}$  was measured from SCG neurons cultured for 5 d, using the amphotericin-B perforated-patch technique (Horn and Marty, 1988; Rae et al., 1991). Patch electrodes (1.5–4 M $\Omega$ ) were filled by dipping the tip for 40 sec into filtered internal solution that comprised (in mM): potassium acetate 80, KCl 30, HEPES 40, MgCl<sub>2</sub> 3 (adjusted to pH 7.3–7.4 with KOH, and 280 mOsmol/l with K acetate). The pipette was then back-filled with internal solution containing 0.07–0.1 mg/ml amphotericin-B. High resistance seals (>2 G $\Omega$ ) were initially achieved, and after amphotericin-B permeabilization, access resistances were generally <25 M $\Omega$ . SCG neurons were perfused at 5–10 ml/min at 32°C with an external solution consisting of (in mM): NaCl 120, KCl 3, HEPES 5, NaHCO<sub>3</sub> 23, glucose 11, MgCl<sub>2</sub> 1.2, CaCl<sub>2</sub> 2.5, and tetrodotoxin (TTX) 0.0005, pH 7.4. Cells were voltage-clamped at approximately –25 mV to preactivate  $I_{K(M)}$  using a switching amplifier (Axoclamp-2A; Axon Instruments, Foster City, CA; switching frequencies 3–5 kHz, filter 0.1 kHz).  $I_{K(M)}$  was measured from the slow deactivation relaxation after a 1 sec jump to a command potential of approximately –55 mV (Haley et al., 1998a), and inhibition was measured as the fractional reduction in the amplitude of this deactivation relaxation in response to either cumulatively increasing concentrations of oxotremorine methiodide (Oxo-M; Research Biochemicals, Natick, MA) or a single application of 1 nM BK (Bachem, Torrance, CA) (see Fig. 2). Data were collected and analyzed using pClamp6 software (Axon Instruments) and expressed as mean  $\pm$  SEM. Statistical analysis of the Oxo-M dose–response curves used two-way ANOVA comparing all treatments across all concentrations of agonist. If a significant effect of treatment was found overall, further analysis was performed using two-way ANOVA to determine which treatment groups contributed to this significance. The bradykinin data were analyzed using Student's *t* test with Welch's correction. *p* values < 0.05 were considered significant.

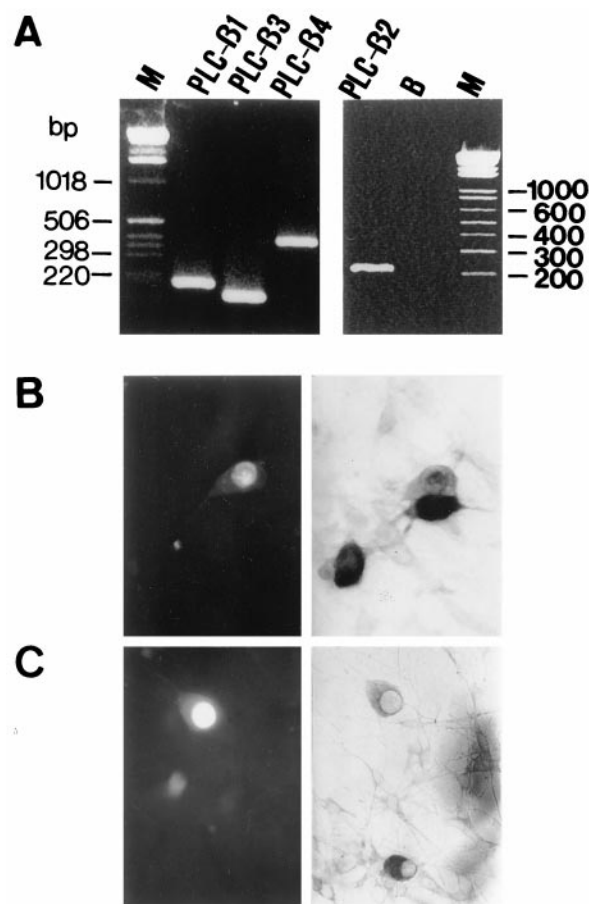
**Reverse transcription PCR.** RNA was extracted from rat SCGs using RNazol B (Biogenesis Ltd.) and reverse-transcribed using oligo-dT and mouse murine leukemia virus reverse transcriptase (Promega, Madison, WI). The oligonucleotide primers used in the PCR were those that were deemed least conserved among the different PLC $\beta$  isoforms to ensure specificity in the amplification. The primers are as follows (“u” denotes sequence in the 3' untranslated region, “s” denotes sense primer, and “a” denotes antisense primer): PLC $\beta$ -1 u22 s/u199a; PLC $\beta$ -2 3111 s/3319a; PLC $\beta$ -3 3564 s/u45a; PLC $\beta$ -4 u5 s/u366a. Cycling conditions were 95°C for 5 min and then 35 cycles of 95°C for 30 sec, 58°C for 30 sec, and 72°C for 1 min followed by a final extension step of 72°C for 10 min.

**Immunocytochemistry.** SCG neurons, cultured and injected as described above, were fixed in acetone and stained using specific polyclonal antibodies against PLC- $\beta$ 1 (sc-205), PLC- $\beta$ 2 (sc-206), PLC- $\beta$ 3 (sc-403), and PLC- $\beta$ 4 (sc-404) (Santa Cruz Biotechnology, Santa Cruz, CA) diluted either 1:1000 or 1:500. Specificity of the antibodies was confirmed by pre-absorbing the antibody with 6- to 10-fold excess (by weight) of the relevant immunogenic peptides (also from Santa Cruz Biotechnology). All dishes of SCG neurons recorded in the electrophysiology experiments were subsequently fixed and stained. The alkaline phosphatase substrate system used was 5-bromo-4-chloro-3-indoxyl phosphate and nitroblue tetrazolium chloride (BCIP/NBT) (Dako, Carpinteria, CA). Because the purple–blue product was too dark to quantitate photometrically, we have assessed whether there was an overall qualitative reduction in staining by comparing each injected cell with its nearest uninjected neighbor and determining (by eye) whether the level of staining was equal to or less than that of the uninjected cell. Using this method we have estimated the proportion of cells with a visible reduction in staining (regardless of the magnitude of this reduction) 48 hr after injection of the antisense plasmid.

## RESULTS

### PLC- $\beta$ isoforms expressed in SCG

RT-PCR demonstrated the presence of mRNA for all four isoforms of PLC- $\beta$  (1, 2, 3, and 4) in rat SCG (Fig. 1A) while the protein for all isoforms was detected immunocytochemically in cultured SCG neurons. Intranuclear injection of antisense against PLC- $\beta$ 1 and PLC- $\beta$ 4 resulted in a reduction in the level of staining for the relevant enzyme 48 hr later (Fig. 1B,C). The

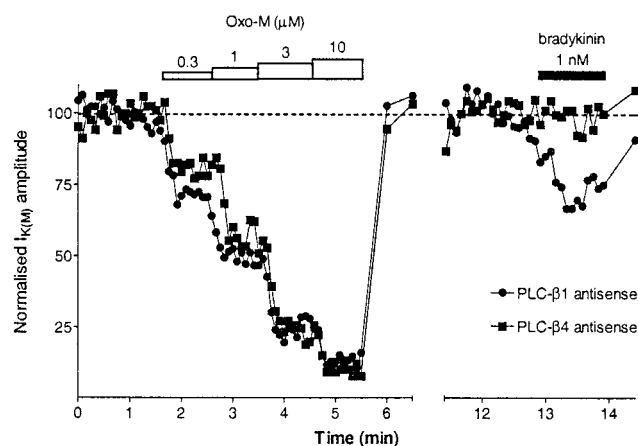


**Figure 1.** A, RT-PCR indicating the presence of mRNA for PLC- $\beta$ 1, PLC- $\beta$ 2, PLC- $\beta$ 3, and PLC- $\beta$ 4 in rat SCG neurons. Lanes labeled M are size markers; lane B is a blank where no template was included in the PCR reaction. B, SCG neuron injected with antisense against PLC- $\beta$ 1 (along with a fluorescent marker; left panel) and stained for PLC- $\beta$ 1 (right panel). C, SCG neuron injected with PLC- $\beta$ 4 antisense and stained for PLC- $\beta$ 4.

PLC- $\beta$ 1 antisense was highly effective and clearly reduced PLC- $\beta$ 1 staining in 37 of 53 neurons (70%; *n* = 7 dishes of cells) without altering levels of PLC- $\beta$ 2 (1 of 12 cells showed reduced staining; 8%; *n* = 3 dishes), PLC- $\beta$ 3 (1 of 12 cells; 8%; *n* = 3 dishes), or PLC- $\beta$ 4 (0 of 13 cells; *n* = 2 dishes). The PLC- $\beta$ 4 antisense was less effective but still reduced visible PLC- $\beta$ 4 staining in 12 of 32 cells (38%; *n* = 10 dishes of cells). Although the PLC- $\beta$ 4 antisense was designed to specifically target the PLC- $\beta$ 4 isoform, it also reduced levels of PLC- $\beta$ 1 (11 of 21 cells; 52%; *n* = 6 dishes) but did not alter PLC- $\beta$ 3 staining (1 of 9 cells; 11%; *n* = 3 dishes) or PLC- $\beta$ 2 staining (1 of 21 cells; 5%; *n* = 2 dishes). Antisense-encoding plasmids were also designed against the remaining PLC- $\beta$  isoforms, but neither the PLC- $\beta$ 2 antisense (2 of 39 cells; 5%; *n* = 5 dishes) nor the PLC- $\beta$ 3 antisense (1 of 9 cells; 11%; *n* = 5 dishes) reduced staining of its respective protein. Electrophysiological data with these ineffective antisense clones is not shown although, as expected, they did not alter  $I_{K(M)}$  modulation by Oxo-M or BK, confirming that injection of DNA plasmids per se does not alter modulation of  $I_{K(M)}$ .

### Effect of PLC- $\beta$ antisense plasmids on $I_{K(M)}$ inhibition by a muscarinic agonist and by bradykinin

Intranuclear injection of antisense against PLC- $\beta$ 1 or PLC- $\beta$ 4 did not alter the amplitude of  $I_{K(M)}$ . Mean values (in picoamperes per



**Figure 2.** Time course of normalized  $I_{K(M)}$  amplitude during cumulatively increasing concentrations of Oxo-M and a single application of bradykinin, as indicated, for neurons injected with PLC- $\beta 1$  and PLC- $\beta 4$  antisense plasmids.  $I_{K(M)}$  was recorded every 5 sec (30 sec during wash-out). Each Oxo-M concentration was applied for 1 min, and bradykinin was applied for 1.5 min. Oxo-M and bradykinin were applied to the same neurons, and wash-out of Oxo-M continued during the break in the x-axis.  $I_{K(M)}$  amplitudes were renormalized before bradykinin application to eliminate any differences in recovery from the Oxo-M application.

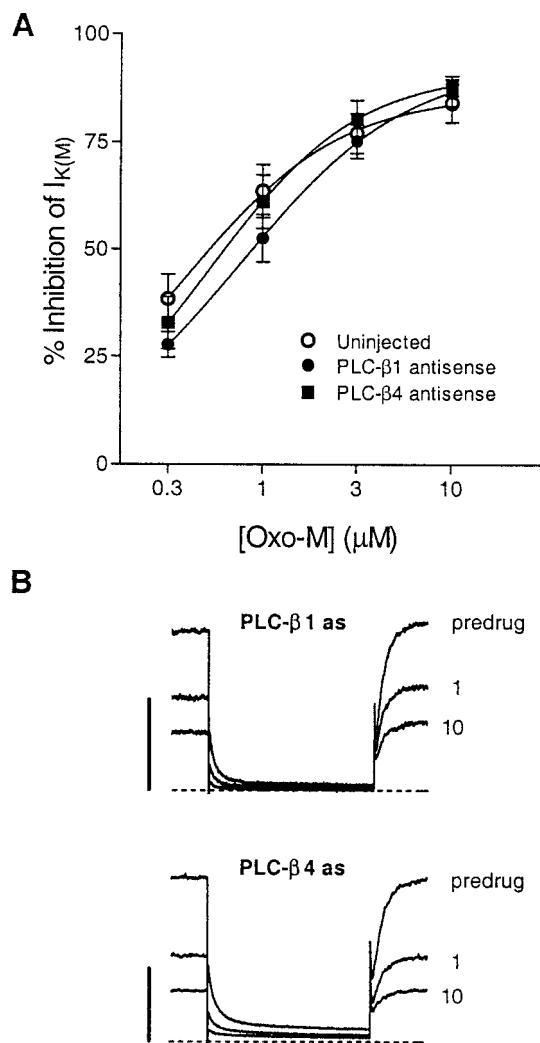
picoFarad)  $\pm$  SEM were: uninjected cells,  $2.91 \pm 0.41$  ( $n = 8$ ); PLC- $\beta 1$  antisense,  $2.39 \pm 0.52$  ( $n = 10$ ); and PLC- $\beta 4$  antisense,  $2.33 \pm 0.36$  ( $n = 10$ ). The resting membrane potential was not altered in cells with reduced PLC- $\beta 1$  or - $\beta 4$  levels (uninjected:  $-58.1 \pm 2.0$  mV,  $n = 10$ ; PLC- $\beta 1$  antisense:  $-60.3 \pm 1.9$  mV,  $n = 13$ ; PLC- $\beta 4$  antisense:  $-58.8 \pm 1.5$  mV,  $n = 11$ ).

Neither antisense affected  $M_1$  mAChR-mediated inhibition of  $I_{K(M)}$  tested 48 hr later (when a reduction in the levels of these enzymes was observed). The dose-response curves to the muscarinic agonist Oxo-M in PLC- $\beta 1$  or PLC- $\beta 4$ -depleted cells were not significantly different from one another or from that in uninjected neurons:  $IC_{50}$  values and Hill slopes were  $0.4 \mu M$  and 1.1 for uninjected neurons,  $0.8 \mu M$  and 1.0 for PLC- $\beta 1$  antisense-injected neurons, and  $0.5 \mu M$  and 1.1 for PLC- $\beta 4$  antisense-injected cells (Figs. 2, 3). In contrast, inhibition by BK was reduced in cells injected with the PLC- $\beta 4$ , but not PLC- $\beta 1$  antisense-expressing plasmid: 1 nM BK produced  $28.5 \pm 6.8\%$  inhibition in uninjected cells ( $n = 8$ ),  $30.8 \pm 7.9\%$  in PLC- $\beta 1$  antisense-injected neurons ( $n = 10$ ), but only  $12.5 \pm 2.0\%$  inhibition in PLC- $\beta 4$  antisense-injected cells ( $n = 10$ ;  $p < 0.05$  compared with uninjected or PLC- $\beta 1$  antisense-injected neurons) (Fig. 4).

## DISCUSSION

The principal inference to be drawn from these experiments is that activation of PLC- $\beta 4$  probably contributes to the inhibition of  $I_{K(M)}$  produced by stimulating BK receptors, but not to that produced by activating  $M_1$  muscarinic acetylcholine receptors. This differentiation accords with, and amplifies, previous conclusions of Cruzblanca et al. (1998) regarding the differential participation of the PLC pathway in M-current inhibition after stimulation of these two G protein-coupled receptors.

RT-PCR clearly demonstrated the presence of mRNA for PLC- $\beta 1$ , - $\beta 2$ , - $\beta 3$ , and  $\beta 4$  in rat SCG. Staining of cultured SCG neurons using specific antibodies also confirmed the presence of these proteins. Using antisense-encoding DNA plasmids we were able to reduce the levels of PLC- $\beta 1$  and PLC- $\beta 4$  in SCG neurons. Although both PLC- $\beta 1$  and PLC- $\beta 4$  have been shown to exist as

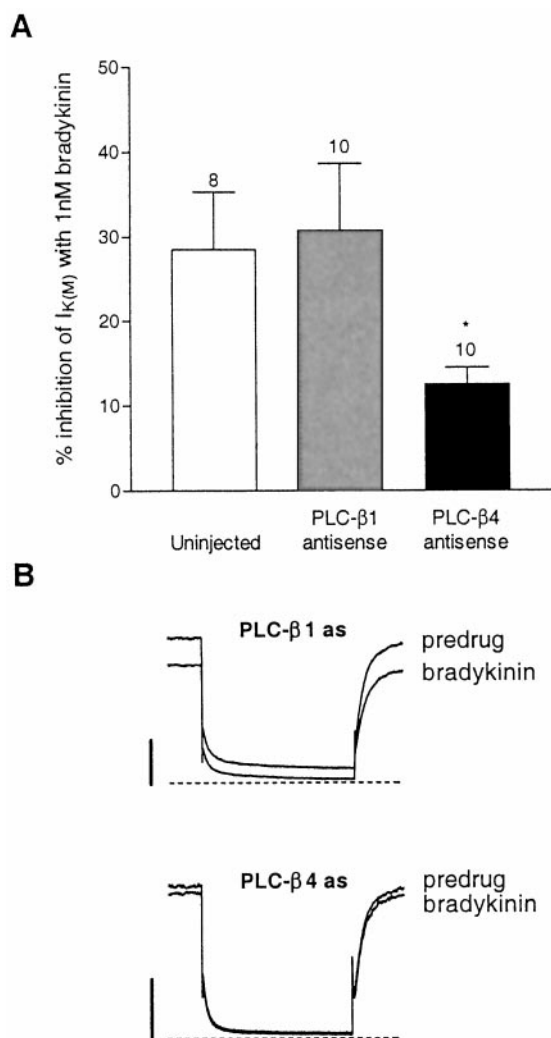


**Figure 3.** PLC- $\beta 1$  and PLC- $\beta 4$  antisense do not alter inhibition of  $I_{K(M)}$  by Oxo-M. **Left panel.** Dose-response curves (mean  $\pm$  SEM, plus best fit curve) for Oxo-M inhibition of  $I_{K(M)}$  in antisense plasmid-injected SCG neurons. There is no significant difference between neurons injected with PLC- $\beta 1$  antisense ( $n = 11$ ), PLC- $\beta 4$  antisense ( $n = 10$ ), and the uninjected neurons ( $n = 10$ ). **Right panel.** Typical  $I_{K(M)}$  deactivation relaxations elicited by a -30 mV step for 1 sec from a holding potential of -25 mV, in the absence and presence of 1 and 10  $\mu M$  Oxo-M for cells injected with either PLC- $\beta 1$  or PLC- $\beta 4$  antisense plasmids (as indicated). The dotted lines represent 0 pA, and the calibration bars indicate 250 pA.

two splice variants (Bahk et al., 1994; Kim et al., 1998), the antisense sequences we have used should target both variants of each enzyme. Indeed, the PLC- $\beta 1$  antisense reduced PLC- $\beta 1$  staining in both the cytosol and the nucleus of cultured SCG neurons (Fig. 1B), suggesting that levels of both splice variants were reduced because, in C6Bu-1 cells, PLC- $\beta 1a$  has been shown to be present mainly in the cytosol, whereas PLC- $\beta 1b$  was present in the nucleus (Bahk et al., 1998). As we found previously with antisense against  $G\alpha_q$  and  $G\alpha_{11}$  (Haley et al., 1998a), not all the antisense sequences designed were effective, and the constructs driving expression of antisense PLC- $\beta 2$  and antisense PLC- $\beta 3$  were unable to reduce staining of their target proteins.

Although the PLC- $\beta 1$  antisense resulted in a robust reduction of PLC- $\beta 1$  levels (Fig. 1B), there was no reduction in either Oxo-M or BK inhibition of  $I_{K(M)}$ . By contrast, the PLC- $\beta 4$  antisense reduced BK inhibition of  $I_{K(M)}$  but left Oxo-M inhibition





**Figure 4.** Inhibition of  $I_{K(M)}$  by bradykinin is attenuated in neurons injected with PLC- $\beta 4$  antisense. **Left panel.** Mean data  $\pm$  SEM for inhibition of  $I_{K(M)}$  by 1 nM BK. Neurons injected with PLC- $\beta 4$  antisense produced significantly less inhibition compared to either uninjected or PLC- $\beta 1$  antisense plasmid-injected neurons ( $*p < 0.05$ ). **Right panel.** Typical  $I_{K(M)}$  waveforms after a 1 sec step in the presence and absence of 1 nM bradykinin with either PLC- $\beta 1$  or PLC- $\beta 4$  antisense plasmid-injected neurons (as indicated). The dotted lines represent 0 pA, and the calibration bars represent 250 pA.

unaltered. This difference between BK and Oxo-M is unlikely to be caused by the fact that Oxo-M is a more “efficacious” inhibitor of  $I_{K(M)}$  (in the sense that it produced a larger maximum inhibition), because the response to even a low concentration (0.3  $\mu$ M) of Oxo-M that matched that produced by BK was unaffected by the PLC- $\beta 4$  antisense (Fig. 3). Also, the concomitant reduction in PLC- $\beta 1$  staining produced by the PLC- $\beta 4$  antisense is unlikely to be responsible for the loss of BK inhibition because the PLC- $\beta 1$  antisense produced a greater and more consistent reduction in PLC- $\beta 1$  protein levels yet did not alter this response. At the very least, therefore, it is reasonable to conclude that BK inhibition of  $I_{K(M)}$  requires PLC- $\beta 4$  activation to a demonstrably greater extent than Oxo-M inhibition. In this respect, our results are in harmony with the conclusions of Cruzblanca et al. (1998) that inhibition of  $I_{K(M)}$  by BK, but not by Oxo-M, involves PLC activation.

In the absence of positive controls, we cannot make any firm

conclusion from the negative effect of PLC- $\beta 1$  antisense—for example, it is possible that sufficient PLC- $\beta 1$  protein remained after antisense depletion to continue to drive  $I_{K(M)}$  inhibition. Likewise, because antisense constructs against PLC- $\beta 2$  or PLC- $\beta 3$  did not reduce the levels of the cognate proteins, we cannot make any conclusions regarding the roles of these two isoforms in  $I_{K(M)}$  inhibition. Nevertheless, a potential selective involvement of PLC- $\beta 4$  in the action of BK would provide an interesting correlation with the results of our previous experiments using overexpressed  $\beta\gamma$  and constitutively active  $\alpha$ -subunits (Haley et al., 1998a), showing that it is the  $\alpha$  subunit of  $G_q$  or  $G_{11}$ , not the  $\beta\gamma$  subunit, that mediates inhibition of  $I_{K(M)}$ , because, of the four isoforms of PLC, it is only PLC- $\beta 4$  that is solely activated by the  $\alpha$ -subunit and not by  $\beta\gamma$ -subunits (Singer et al., 1997).

At first sight, our data (and also those of Cruzblanca et al., 1998) would seem to be in conflict with those of Hildebrandt et al. (1997), who found that an inhibitor of PLC did not alter BK inhibition of the  $I_{K(M)}$ -like current in NG108 cells. Recent advances in our understanding of the channels underlying the  $I_{K(M)}$ , however, provide a possible explanation for this discrepancy. It is now thought that the  $I_{K(M)}$ -like current in NG108 cells actually comprises two separate currents: the first has similarities with  $I_{K(M)}$  in SCG and probably results from KCNQ2/3 channel activity (cf. Wang et al., 1998), whereas the second, slower current, is pharmacologically distinct and probably results from *merg* channel activity (Selyanko et al., 1999). The “SCG-like” (KCNQ)  $I_{K(M)}$  in NG108 cells is sensitive to blockade by TEA (Selyanko et al., 1999); because the experiments presented in Hildebrandt et al. (1997) were all performed in the presence of TEA, they were probably examining BK inhibition of the *merg* based  $I_{K(M)}$ -like current, which may be inhibited by a different mechanism from  $I_{K(M)}$  in SCG.

$I_{K(M)}$  channels in SCG neurons can be inhibited by high concentrations of intracellular  $Ca^{2+}$  (Selyanko and Brown, 1996). Because BK can increase intracellular  $Ca^{2+}$  levels in rat SCG neurons (Cruzblanca et al., 1998; del Rio et al., 1999), it is possible that BK may inhibit  $I_{K(M)}$  by stimulating PLC- $\beta 4$  to generate IP<sub>3</sub> and thereby mobilizing  $Ca^{2+}$  from internal stores (Cruzblanca et al., 1998). We attempted to determine whether BK induced  $Ca^{2+}$  rises in SCG require PLC- $\beta 4$  activation but found that the  $Ca^{2+}$  rises we detected were small and very variable, making assessment of any antisense effect impossible (our unpublished data). M<sub>1</sub> mAChR mediated inhibition of  $I_{K(M)}$  is unlikely to involve  $Ca^{2+}$  as a messenger because agonists at this receptor do not appear to raise internal  $Ca^{2+}$  levels in SCG (Cruzblanca et al., 1998). Even when modest rises in internal  $Ca^{2+}$  levels have been observed (e.g., under conditions where the cells are depolarized) these rises are not correlated with inhibition of  $I_{K(M)}$  (del Rio et al., 1999). Furthermore, buffering of intracellular  $Ca^{2+}$  by the  $Ca^{2+}$  chelator BAPTA attenuates BK inhibition of  $I_{K(M)}$ , but leaves Oxo-M inhibition intact (Cruzblanca et al., 1998).

Thus, both the experiments of Cruzblanca et al. (1998) and those described in the present paper lead to the conclusion that the inhibition of M-current in rat SCG neurons produced by stimulating muscarinic or bradykinin receptors proceeds via different intracellular pathways. Because both receptors can couple to the same family of G-proteins and because both are intrinsically capable of inducing inositol phosphate production within these neurons (del Rio et al., 1999), the reason for this divergence in M channel signaling is not yet clear. Nevertheless, it highlights

the point that M channels may be regulated by more than one mechanism.

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